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Intermittent agitation contributes to uniformity across the bed during pectinase production by *Aspergillus niger* grown in solid-state fermentation in a pilot-scale packed-bed bioreactor

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ABSTRACT

Solid-state fermentation could be used to produce low-cost pectinases that could then be used to saccharify pectin in citrus waste biorefineries. Recently, we produced pectinases in a pilot-scale packed-bed bioreactor, growing *Aspergillus niger* on a substrate mixture consisting of 27 kg of wheat bran and 3 kg of sugarcane bagasse (dry mass). However, the agglomeration of particles and shrinkage of the bed created preferential flow paths, leading to overheating within the bed and poor uniformity of pectinase levels at the end of the fermentation. In the current work, we used intermittent agitation as a strategy to minimize agglomeration, comparing one agitation (10 h), three agitations (at 8, 10 and 12 h) and five agitations (every 2 h from 8 to 16 h). The pectinase activity in the bed was uniform after agitation, but poor uniformity occurred when the bed was left unmixed for more than 10 h. The best regime was that with three agitations: For 15 samples removed from different vertical and horizontal positions of the bed at 20 h, the average pectinase activity was 22 U g^{-1}, with a sample standard deviation of 2 U g^{-1}. We conclude that the use of intermittently-mixed packed-bed bioreactors is a promising strategy for producing pectinases in solid-state fermentation.

1. Introduction

Several authors have recently suggested the possibility of establishing biorefineries to produce a range of products from citrus wastes, including limonene, pectin, ethanol and biogas [1,2]. However, since the amount of pectin contained in citrus wastes is several-fold greater than the world pectin market [3], it would be more interesting to saccharify the pectin to liberate D-galacturonic acid, which constitutes over 75% of the polymer [4]. D-galacturonic acid could then be converted to mucic acid and L-galactonic acid, which have several current or potential commercial applications and can also act as precursors of other products such as ascorbic acid, bioplastics, mucic acid and L-galactonic acid [5–7].

For the saccharification of pectin, enzymatic hydrolysis is preferable to acid hydrolysis since it preserves the D-galacturonic units that are released. This is unlike acid hydrolysis, for which processes that are strong enough to hydrolyze the glycosidic bonds between the D-galacturonic acid residues in pectin also degrade a significant proportion of the D-galacturonic that is released [8]. However, in order for enzymatic hydrolysis to be viable, it is essential to have low-cost pectinases.

One possible strategy for producing low-cost pectinases is to use solid-state fermentation (SSF) [9]. However, there are no general rules for guiding scale-up of SSF bioreactors [10] and it is therefore necessary to scale-up processes on a case-by-case basis. In the case of pectinases, a scale-up study was recently initiated by Pitol et al. [9]. Their work represents the largest pilot-scale study of pectinase production by SSF to date: the filamentous fungus *Aspergillus niger* was cultivated on 27 kg of wheat bran, with the addition of 3 kg of sugarcane bagasse as a bed porosity modifier (dry masses). The bioreactor was operated as a packed-bed with forced aeration. The

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bed remained completely static during the fermentations and, as a result, problems were encountered. For example, in their third pilot-scale fermentation, the fungus knitted the substrate particles into agglomerates and the bed shrank, producing gaps between the bed and the bioreactor wall. When this happened, the air flowed through these gaps rather than through the bed itself. As a consequence, the bed overheated, with temperatures as high as 43 °C being measured at the top of the bed [9]. At the end of the fermentation, the pectinase activities measured in different locations of the bed were quite different, varying from 11 to 28 U per gram of dry substrate.

The problem of substrate agglomeration by filamentous fungi in SSF was investigated by Schutyser et al. [11], who studied the growth of *Aspergillus oryzae* on wheat grains in a horizontal drum. In their studies, the bed was initially left static and then later agitated with the intention of breaking the agglomerates apart. They concluded that, if the bed is left too long before the first agitation event, large agglomerates will form in which the "mycelial bonds" between the particles are sufficiently strong to remain intact when the bed is mixed by rotating the drum. They suggested that the best strategy to prevent the formation of unbreakable agglomerates is to use an agitation event early in the fermentation to disrupt the mycelial bonds between the particles before they become too strong. Although their experimental investigations were limited to a single preemptive agitation event, they did raise the possibility that it might be necessary to have subsequent agitation events to maintain the substrate bed free of agglomeration problems throughout the whole process.

The recommendations made by Schutyser et al. [11] regarding the use of preemptive agitation events to prevent substrate agglomeration prompted us to investigate whether this strategy would mitigate the agglomeration problems reported by Pitol et al. [9] for the cultivation of *Aspergillus niger* on the mixture of wheat bran and sugarcane bagasse in the pilot-scale bioreactor.

2. Materials and methods

The system studied in the current work is the same as that studied by Pitol et al. [9], namely the growth of *Aspergillus niger* CH4 (kindly provided by Dr. Jesus Cordova and Prof. Dr. Gustavo Viniegra-Gonzalez) on a substrate mixture consisting of 27 kg of wheat bran and 3 kg of sugarcane bagasse (dry mass), in a pilot-scale packed-bed bioreactor with a bed capacity of 200 L. This bioreactor can be agitated intermittently by rotation around its central axis. Note that moisture contents are reported throughout this paper as %w/w, on a wet basis. They were determined in an infrared balance (Gehaka IV 2000, São Paulo, Brazil).

2.1. Source and preparation of raw materials

The sugarcane bagasse was donated by Usina de Álcool Melhoramentos (Jussara, Brazil). It was sun-dried in the open air for several days before use. Otherwise, it was used as received (i.e. without milling or sieving). The wheat bran was donated by Anaconda Industrial e Agrícola de Cereais (Curitiba, Brazil) and used as received.

2.2. Pilot-scale packed-bed bioreactor

The bioreactor consists of an AISI 306 stainless steel cylinder with a perforated plate (70 cm × 60 cm) at the bottom, which supports the substrate bed (Fig. 1 a and b) [9,12]. A wire mesh with 1-mm apertures covers the base plate. The air is injected below the base plate and flows from the bottom to the top of the bed.

The air preparation and temperature measurements are the same as for Pitol et al. [9] and Biz et al. [12]. A blower captures air from outside the building and blows it through a glass wool filter and into the bottom of a humidification tower. The flow is measured by a differential pressure flow meter (Kobold, Hofheim, Germany). Water is pumped from a 1200-L reservoir (kept at 32 ± 2 °C) into the top of the humidification tower and drains back into the reservoir. The air exits the top of the humidification column with a relative humidity of 99% and a temperature close to the water reservoir temperature. By the time that it enters the bottom of the bioreactor, it is about 2 °C cooler than when it left the humidification column. The outlet air passes through a gas washer. Thermocouples measure the inlet and outlet air temperatures. There are also four thermocouple arrays within the bioreactor, positioned at heights of 5, 18, 33 and 46 cm (Fig. 1b, items 2, 3, 4 and 5, respectively). Each array measures the temperature at four different horizontal positions. Temperatures reported for each bed height represent the averages of these four measurements on the horizontal.

2.3. Preparation of inoculum for the pilot-scale bioreactor

The inoculum was prepared in the same manner as that described by Pitol et al. [9]. Fresh cultures of *Aspergillus niger* CH4 were grown on potato dextrose agar at 30 °C for 4 days to allow sporulation. The spores were suspended in sterile distilled water and the suspension was filtered through sterile gauze to remove any mycelium. Spores were counted in a Neubauer chamber and the concentration in the suspension was adjusted to 2×10^7 spores mL^{-1}. This preparation is denoted as "pre-inoculum".

Forty 250-mL Erlenmeyer flasks, each containing 10 g (dry mass) of a mixture of 30% sugarcane bagasse and 70% wheat bran (w/w), were autoclaved (121 °C, 15 min). $(NH_4)_2SO_4$ solution was autoclaved (121 °C, 15 min) and added to each flask to obtain a final moisture content of 50% (w/w, wet basis) and a final concentration of $(NH_4)_2SO_4$ of 4.4% (w/w, based on total dry substrate). Pre-inoculum was added to give 10^7 spores per gram of dry substrate and the flasks were incubated at 30 °C for 7 days to allow sporulation. The spores were suspended in sterile distilled water and then filtered in a funnel with sterile gauze to remove residual substrate. The spore concentration in the suspension was determined using a Neubauer chamber. The spore concentration in the suspension was adjusted to 2×10^7 spores mL^{-1}. This preparation is denoted as "pilot-inoculum".

2.4. Solid-state fermentation in the pilot-scale bioreactor

Except for the fact that the bed was intermittently agitated, the pilot-scale bioreactor was operated as described by Pitol et al. [9]. The air flow rate was kept constant in all fermentations at 150 $m^3\,h^{-1}$, resulting in a superficial air velocity of 0.1 $m\,s^{-1}$. All fermentations were carried out with 27 kg of wheat bran and 3 kg of sugarcane bagasse (dry masses), which resulted in an initial bed height of 40 cm. The solid substrate, sufficient solution of $(NH_4)_2SO_4$ to give an initial concentration of 4.4% (w/w, based on total dry substrate) and the additional water needed to obtain an initial moisture content of 62% were autoclaved (121 °C, 2 h). After cooling, sufficient pilot-inoculum was added to give 2×10^7 spores per gram of dry substrate and then the inoculated solid was added to the bioreactor, which was rotated for 30 min to homogenize the bed. After this period, the bed surface was leveled using a rake, the bioreactor was closed and the air supply system was connected. The bioreactor was left static for most of the time. For each agitation event, the air inlet, the air outlet and the thermocouple cables were disconnected and then the bioreactor was rotated around its central axis at 5 rpm for 2 min. After this, the top of the bed was again leveled with a rake. The inlet and outlet air lines and cables were then reconnected.